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*January 03, 2005*

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**APPLICATION NUMBER: 60/513,231**

**FILING DATE: *October 21, 2003***

**RELATED PCT APPLICATION NUMBER: *PCT/US04/34915***



Certified By

Jon W Dudas

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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102103

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Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input type="checkbox"/> Customer Number: <span style="border: 1px solid black; display: inline-block; width: 200px; height: 20px; vertical-align: middle;"></span>					
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ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		<b>23</b>		<input type="checkbox"/> CD(s), Number _____	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		<b>11</b>		<input type="checkbox"/> Other (specify) _____	
<input type="checkbox"/> Application Date Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE Amount (\$)	
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees.				<div style="border: 1px solid black; width: 100px; height: 50px; margin: 0 auto;"></div>	
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input type="checkbox"/> No.					
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[Page 1 of 2]

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

**Daniel M. Chambers**

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**658.350.9690**

Date

**21 OCT 2002**

REGISTRATION NO.

**34,581**

(if appropriate)

Docket Number:

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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17638 U.S. PTO

PTO/SB/17 (10-03)

Approved for use through 07/31/2006. OMB 0651-0032

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# FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT

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60.00

## Complete if Known

Application Number

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Filing Date

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First Named Inventor

Albani, S.

Examiner Name

Not yet assigned

Art Unit

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Attorney Docket No.

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## METHOD OF PAYMENT (check all that apply)

☒ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None☐ Deposit Account:Deposit  
Account  
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The Director is authorized to: (check all that apply)

☐ Charge fee(s) indicated below ☐ Credit any overpayments☐ Charge any additional fee(s) or any underpayment of fee(s)☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

## FEE CALCULATION

## 1. BASIC FILING FEE

Large Entity		Small Entity		Fee Description	Fee Paid
Fee Code	Fee (\$)	Fee Code	Fee (\$)		
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80
SUBTOTAL (1)				(\$)	60

## 2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims	Fee from below	Fee Paid
Total Claims	-20** =	X	
Independent Claims	-3** =	X	
Multiple Dependent			

Large Entity		Small Entity		Fee Description
Fee Code	Fee (\$)	Fee Code	Fee (\$)	
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$)

0

\*\*or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

## 3. ADDITIONAL FEES

Large Entity Small Entity

Fee Code	Fee (\$)	Fee Code	Fee (\$)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for ex parte reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify)

\*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$)

0

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21 OCT 2003

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# TOLL-LIKE RECEPTOR BINDING PEPTIDES AND METHODS OF USE

## FIELD OF THE INVENTION

This invention relates to novel peptides and methods of use thereof. In particular, there is provided novel toll-like receptor (TLR) binding peptide molecules useful in modulating inflammatory responses in immune-mediated diseases, ranging from autoimmunity to cancer to infectious diseases. More specifically, this invention relates to selecting heat shock proteins (HSP)-derived peptides capable of interfering with or modulating (e.g., inhibiting) proinflammatory responses resulting from and/or related to the interaction between HSPs and derivatives thereof and TLRs on immune cells.

## BACKGROUND OF THE INVENTION

### Heat Shock proteins and Toll Like Receptors

HSPs are highly conserved molecules that act as chaperones involved in the folding of newly synthesized proteins. In addition, they transport antigens and, after internalization, they mediate antigen-specific cytotoxic T cell, as well as Th cell-dependent, responses. During stress, synthesis of HSPs is up-regulated in prokaryotic, as well as in eukaryotic, cells. Moreover, intracellular redistribution of HSPs, and the expression of these molecules on the cell surface, has been reported.

Recently, the stimulatory capacity of extracellular HSP60 and HSP70 on the innate immune system has been recognized. Human and murine macrophages respond to both bacterial and human HSP60 with the release of proinflammatory mediators such as TNF or IL-6 and of the Th1-promoting cytokines IL-12 and IL-15, suggesting that HSP60 might act as a "danger signal" for the innate immune system. The cell surface receptor for HSP60 has not yet been completely characterized. Increased local expression of HSP60, and presumably other HSPs, in target tissues has been found in various inflammatory diseases such as rheumatoid arthritis, insulinitis, and atherosclerosis. It has been reported that HSP60 is an important target for immune responses during chronic inflammation or atherosclerosis. T cell responses to autologous HSP60 therefore have been suggested to regulate inflammatory or auto-aggressive immune reactivities.

There is evidence that CD14 and TLR 2 and 4 are involved in HSP60-mediated cell activation. However, binding to macrophages appears to be TLR4-independent. Recently, a report on HSP60-induced signaling pathways in macrophages has been published, suggesting that the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), as well as I B kinase, may be activated after endocytosis of HSP60 by murine macrophage. These kinases induce transcription factors like NF-KB that are responsible for cytokine (e.g., TNF) gene transcription.

Interestingly, HSP60 and 70 are also antigens for the adaptive immune system, and T cell responses to epitopes from these HSPs regulate inflammatory diseases like rheumatoid arthritis, insulin dependent diabetes mellitus, and atherosclerosis.

Indeed, autologous HSP60 has been suggested to control the balance of T cell responses in inflammation and autoimmunity. Activation of T cells is induced through their interaction with antigen presenting cells (APCs), for example, dendritic cells (DCs). DCs reside in an immature form in non-lymphoid tissues where they efficiently capture foreign antigens. Upon activation by pathogens or by inflammatory stimuli like TNF or IL-1, DCs migrate to lymphoid organs where they potently activate antigen-specific T cell responses. During migration, DCs undergo functional and phenotypic changes termed as "maturation." For example, they up-regulate surface molecules like MHC class I and II molecules, CD86, CD40, and CD54, and, thus, become potent inducers of T cell activation. Maturation is further driven by CD40-CD40 ligand interaction upon contact of DCs with T cells. In parallel, DCs lose their capacity for endocytosis and de novo MHC class II synthesis during migration to the lymphoid tissues. This feature enables DCs to preserve the antigen taken up in the periphery for antigen-specific T cell activation. In addition, mature DCs are able to release large amounts of proinflammatory cytokines like TNF or IL-1 and of the Th1-promoting cytokines IL-12 and IL-18 but they also secrete counter regulatory IL-10. Therefore, DCs are not only unique in induction of naive T cell activation, but also play a decisive role in Th cell polarization.

Turning now to TLR subject matter, recognition of pathogens is also mediated by a set of germ line-encoded receptors that are referred to as pattern-recognition receptors (PRRs). These receptors recognize conserved molecular patterns (pathogen-associated

molecular patterns), which are shared by large groups of microorganisms. We have discovered that TLRs function as the PRRs in mammals and play an essential role in the recognition of microbial components. The TLRs may also recognize endogenous ligands induced during the inflammatory response. Similar cytoplasmic domains may allow TLRs to use the same signaling molecules used by the interleukin 1 receptors (IL-1Rs); these include MyD88, IL-1R-associated protein kinase and tumor necrosis factor receptor-activated factor 6. Evidence is accumulating that the signaling pathways associated with each TLR are not all identical and may, therefore, result in different biological responses. Thus, there has been no disclosure of using TLRs in a specific and deliberate manner to affect disease states in the medical field.

Therefore, there is a need in the art for inventions such as that described herein, which provides a use for peptides derived from various peptides, e.g., HSP60, to interact with TLRs for the medical purpose of modulating immune response in patients.

## SUMMARY OF THE INVENTION

In a first aspect, isolated TLR-binding peptides are provided. In certain preferred embodiments, the peptides of the invention comprise core TLR-binding motifs. In further related embodiments, these motifs comprise amino acid sequences of human and bacterial HSP (e.g., hsp60) proteins. The peptides of the invention provide isolated amino acid molecules comprising polypeptides having at least about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity, and most preferably at least about 95% sequence identity with a TLR-binding peptide that comprises an amino acid sequence according to any of the sequences of SEQ ID NOS:1-54.

In a second aspect, the invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a peptide having at least about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity, and most preferably at least about 95% sequence identity with a TLR-binding peptide of the invention. Of course, the peptides of the invention can be made by other techniques, including peptide synthetic chemistries (e.g., solid state peptide synthesis).

In a third aspect, the invention provides for TLR-binding peptide mimetic molecules useful in modulating the natural activity of the TLR receptor in immune regulation. Relatedly, the peptides and mimetics of the invention can be incorporated into various compositions, including pharmaceutical compositions wherein a peptide or peptide mimetic is combined with a pharmaceutically acceptable carrier. Such compositions can be liquid or dry, and can optionally include excipients and other molecules.

In a fourth aspect, the TLR-binding peptides and mimetic molecules of the invention are useful in methods of modulating immune responses in immune-mediated diseases. For example, the peptides and mimetic molecules of the invention can be used in immune modulation and thus can provide for enhancing innate immune responses, such as, for example, inducing or enhancing inflammatory responses. With respect to such embodiments, the enhancement can be used, for example, to provide for clearance of infectious agents, cancer cells, or to induce or enhance response to a vaccine.

Alternatively, the peptides can be used as competitive inhibitors of a natural ligand for a TLR. In this regard, the peptides and mimetic molecules of the invention can be used to bring about down regulation of an immune pathway. For example, down regulation can comprise down regulation in autoimmunity such as in Rheumatoid Arthritis (RA), Juvenile Idiopathic Arthritis (JIA), Psoratic Arthritis (PA), Osteoarthritis (OA), Inflammatory Bowel Disease (IBD), Multiple Sclerosis (MS), and Dermato Myositis (DM).

The peptides and peptide mimetics of the invention are also contemplated to be used to treat cancerous conditions such as melanoma, leukemia, lymphoma, solid tumours (lung, liver, kidney, brain, bladder), retinoblastoma, sarcomas and other connective tissue cancers, and the like. The molecules of the invention are also contemplated for use in treating immune-mediated pathogenic infections including such immune mediated microbial infections as tuberculosis, leprosis, bacterial infections of Gram positive and Gram negative microorganisms, HIV/AIDS, Epstein Barr Virus and Cytomegalovirus infections, and protozoan infections, such as Leishmania, and the like.

Use of the peptides and mimetics of the invention are also contemplated in Insulin-Dependent Diabetes Mellitus (IDDM), Systemic Lupus Erythematosus (SLE), Sjogren's

Syndrome, Scleroderma, Polymyositis, Chronic Active Hepatitis, Mixed Connective Tissue Disease, Primary Biliary Cirrhosis, Pernicious Anemia, Autoimmune Thyroiditis, Idiopathic Addison's Disease, Vitiligo, Gluten-Sensitive Enteropathy, Graves' Disease, Myasthenia Gravis, Autoimmune Neutropenia, Idiopathic Thrombocytopenia Purpura, 5 cirrhosis, pemphigus vulgaris, autoimmune infertility, Goodpasture's Disease, Bullous Pemphigoid, Discoid Lupus, Ulcerative Colitis, and Dense Deposit Disease). The diseases set forth above, as referred to herein, include those exhibited by animal models for such diseases, such as, for example Non-Obese Diabetic (NOD) mice for IDDM and Experimental Autoimmune Encephalomyelitis (EAE) mice for multiple sclerosis.

10 In a fifth aspect, the peptides and mimetics of the invention can be used to modulate specific immune responses such as a TH-1 response (i.e., a stimulating response wherein proinflammatory cytokines are induced, such as  $\text{TNF}\alpha$  and  $\text{INF}\gamma$ , IL-6, IL-2, IL-12), or the T cells that have a regulatory function are induced to bring about either a TH2 or TH3 response (i.e., a regulatory response wherein anti-inflammatory 15 cytokines are induced, such as  $\text{TGF}\beta$ , IL-4, and IL-10).

In a sixth aspect, the peptides and mimetics of the invention in some form affect, or alternatively are associated with, activities, or lack thereof, of cytokines and other immune mediators, including the following:

***Pro-inflammatory responses***  
20 IL-1a/b (interleukin 1);  $\text{TNF-}\alpha$  (tumor necrosis factor alpha); LT (lympotoxin); IL-6 (interleukin6); GM-CSF (granulocyte macrophage colony stimulating factor); M-CSF (macrophage colony stimulating factor); LIF (leucocyte inhibitory factor); Oncostatin M (oncostatin M); IL-2 (interleukin 2); IL-3 (interleukin 3); IL-7 (interleukin 7); IL-9 (interleukin 9); IL-12 (interleukin12); IL-15 (interleukin 15);  $\text{IFN}\alpha/\beta$  (interferon 25 alpha/beta);  $\text{IFN-}\gamma$  (interferon gamma); IL-17 (interleukin 17); IL-18 (interleukin 18).

***Immunoregulatory responses***  
IL-4 (interleukin 4); IL-10 (interleukin 10); IL-11 (interleukin 11); IL-13 (interleukin 13);  $\text{TGF-}\beta$  (transforming growth factor beta).

***Chemokines responses***  
30 IL-8 (interleukin 8); Groa (melanoma growth stimulating activity); MIP-1 (macrophage inflammatory protein); MCP-1 (monocyte chemoattractant protein); ENA-78 (epithelial

neutrophil activating peptide 78); RANTES (regulated upon activation T cell expressed & secreted).

### ***Mitogens***

FGF (Fibroblast growth factor); PDGF (Platelet-derived growth factor); VEGF (vascular  
5 endothelial growth factor).

In a related aspect, such cytokines and immune mediators can be blocked by TLR-binding peptides of the invention.

In still another aspect, the invention also provides a novel methodology for  
10 determining likely TLR-binding peptide motifs by performing *in silico* computer modeling comprising methods based on, among other factors, the sequence and structure of the peptides of the invention.

In yet further embodiments, the peptides of the invention are contemplated to be used in diagnostic and in therapeutic applications for patients with autoimmune diseases  
15 including cancers and infectious diseases.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows an Antigenic Index graph that shows regions of the protein predicted to be exposed on the protein surface plotted above the line and unexposed  
20 regions below the line.

Figure 2 shows a Kyte/Doolittle hydrophilicity graph showing average hydrophilicity values above the axis and hydrophobic regions below the line.

Figure 3 shows Surface Probability wherein plots above the line approaching a value of 1 represents a hexapeptide centered at such point is predicted to have exposure at  
25 the surface of the protein.

Figure 4 shows a Chou-Fasman chart indicating regions of helix vs. sheets and turns.

Figure 5 shows an Antigenic Index which plot is smoothed by the refinement of the invention.

Figure 6 shows a Hydrophilicity plot which plot is smoothed by the refinement of  
30 the invention.

Figure 7 shows a Surface Probability plot which plot is smoothed by the refinement of the invention.

Figure 8 shows a Chou-Fasman plot which plot is smoothed by the refinement of the invention.

5        Figure 9 is a three-dimensional stick model of HSP60 from *E. coli*. A predicted surface region is noted in yellow corresponding the region 426-444 of the *E. coli* HSP60 protein, one of the selected peptides.

10       Figures 10A-D are a series of graphs showing the effect of different peptides on expression of TLR2, TLR4 receptor, and secretion of TNF-alpha and IL-6 proinflammatory cytokines, on monocyte-derived dendritic cells.

15       Figure 11 is a graph showing the effect of different peptides on TNF- $\alpha$  secretion from a mouse macrophage cell line, RAW264.7. Cell preparation and stimulation for TNF- $\alpha$  production were seeded into flat-bottomed 96-well plates ( $0.1 \times 10^6$  cells/200ml well). After 18 hrs of incubation, cells were stimulated with the different peptides and, as a control LPS was used. Supernatants were harvested respectively at 6, 24, 48, and 72 hours. ELISA assay was used and the amount of TNF-alpha in culture supernatants was quantified using a Mouse TNF- $\alpha$  Enzyme-Linked ImmunoSorbent Assay. TNF- $\alpha$  values were extrapolated using a standard curve. Results are expressed in picograms TNF- $\alpha$ /ml. Values shown were calculated by subtracting for each peptide the value of unstimulated cells. LPS data not shown.

20       Figure 12 is a diagram showing the general interconnection of cytokine stimulation/expression between innate and adaptive immunity pathway.

      Figure 13 is a flow chart outlining the experimental pathway for Examples 2 and 3, below.

25       Figure 14 is a graph showing TNF- $\alpha$  production in dendritic cells using real time PCR.

      Figures 15A-B are graphs showing percentage of CD8+ cells and TNF- $\alpha$  production in dendritic cells (CD83+, HLA-DR+) measured with staining.

## DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, peptides comprising core TLR-binding motifs are provided. Such motifs may be found in various heat shock proteins, including hsp-60, hsp-70, and dnaJ proteins derived from organisms including *E. coli* (e.g., strain K12), *Mycobacterium tuberculosis*, and humans.

Table 1

Peptide source	Amino Acid Position	Amino Acid Seq.	Peptide reference number	Seq. Id. No.
Human hsp 60	156-162	KKQSKPVT	P22	22
	154-168	ELKKQSKPVTPEEI	P27	27
	230-241	NTSKGQKCEFQD	P28	28
	300-320	VKAPGFGDNRKNQLKDMAIAT	P3	2
	302-314	APGFGDNRKNQLK	P29	29
	360-375	GKGDKAQIEKRIQEI	P23	23
	360-376	GKGDKAQIEKRIQEIE	P30	30
	380-400	VTTSEYEKEKLNERLAKLSDG	P4	4
	381-398	TTSEYEKEKLNERLAKLS	P31	31
	382-396	TSEYEKEKLNERLAK	P24	24
<i>E. coli</i> hsp 60	547-558	TEIPKEEKDPGM	P32	32
	195-211	FDRGYLSPYFINKPETG	P33	33
	345-375	GVAQIRQQIEEATSDYDREKLQERVAKLAGG	P2	6
	346-372	VAQIRQQIEEATSDYDREKLQERVAKL	P34	34
	348-370	QIRQQIEEATSDYDREKLQERVA	P35	35
	387-396	VEMKEKKARV	P36	36
	388-396	EMKEKKAR	P25	25
	426-444	LADLRGQNEQNVGIKVAL	P1	8
	429-439	LRGQNEQNVG	P37	37
	95-110	DIQQRYPHLPYQFQAS	P38	38
<i>E. coli</i> hsp 70	96-117	IQQRYPHLPYQFQASENGLPMI	P7	1
	159-172	YFDDAQRQGTQDAA	P39	39
	249-268	IREQAGIPDRSDNRVQRELL	P8	3
	253-266	AGIPDRSDNRVQRE	P40	40
	583-596	QAIKNVDKQTQDFA	P41	41
	59-68	IELEDPYEKI	P42	42
<i>Mycobacteria</i> hsp 60	133-143	GAKEVETKEQI	P43	43
	135-143	KEVETKEQ	P26	26
	204-213	TDPERQEAVL	P44	44
	342-370	GRVAQIRQEIENSDDYDREKLQERLAKL	P5	10
	343-370	RVAQIRQEIENSDDYDREKLQERLAKL	P45	45
	346-367	QIRQEIENSDDYDREKLQERL	P12	11
	346-368	QIRQEIENSDDYDREKLQERLA	P46	46
	383-402	TEVELKERKHRIEDAVRNAG	P6	12

	385-399	VELKERKHRIEDAVR	P47	47
	386-394	ELKERKHRI	P13	13
	518-529	VADKPEKEKASV	P48	48
			P49	49
Human hsp 70	28-41	IIANDQGNRTTPSY	P50	50
	239-261	NHFVEEFKRKHKKDISQNKRAVR	P9	5
	240-265	HFVEEFKRKHKKDISQNKRAVRRLRT	P14	14
	244-257	EFKRKHKKDISQNK	P51	51
	244-261	EFKRKHKKDISQNKRAVR	P52	52
	262-279	RLRTACERAKRTLSSSTQ	P53	53
	414-427	IKRNSTIPTKQTQI	P54	54
	490-500	ATDKSTGKANK	P10	7
	501-519	ITITNDKGRLSKEEIERMV	P55	55
	502-545	TITNDKGRLSKEEIERMVQEAKEYKADEVQ RERSAKNALESY	P56	56
	509-545	RLSKEEIERMVQEAKEYKADEVQRERSA KNALESY	P57	57
	509-542	RLSKEEIERMVQEAKEYKADEVQRERSA KNAL	P15	15
	510-532	LSKEEIERMVQEAKEYKADEVQ	P58	58
	561-575	KISEADKKKVLDKCQ	P16	16
	564-574	EADKKKVLDKC	P11	9
	580-602	WLDANTLAEKDEFEHKRKELEQV	P59	59
	586-601	LAEKDEFEHKRKELEQ	P17	17
	587-599	AEKDEFEHKRKEL	P60	60
Human dnaj	14-26	GASDEEIKRAYRR	P18	18
	18-25	EEIKRAYR	P61	61
	25-39	RRQALRYHPDKNKEP	P62	62
	38-50	EPGAEEKFKEIAE	P63	63
	54-70	VLSDPRKREIFDRYGEE	P64	64
	144-164	VNFGRSRSAQEPARKKQDPPV	P65	65
	176-203	YSGCTKKMKISHKRLNPDGKSIRNEDKI	P66	66
	299-314	GEGLPLPKTPEKRGDL	P67	67
<i>E. coli</i> dnaj	11-35	GVSKTAEEREIRKAYKRLAMKYHPD	P19	19
	15-26	TAEEREIRKAYK	P68	68
	15-32	TAEEREIRKAYKRLAMKY	P69	69
	34-66	PDRNQGDKEAEAKFKEIKEAYEVLTDQKR AAY	P70	70
	39-66	GDKEAEAKFKEIKEAYEVLTDQKRAAY	P20	20
	41-54	KEAEAKFKEIKEAY	P71	71
	193-213	IKDPCNKCHGHGRVERSKTLS	P72	72
	333-346	LNERQKQLLQELQE	P21	21
	334-345	NERQKQLLQELQ	P73	73
	345-365	QESFGGPTGEHNSPRSKSFFD		

*E. coli* HSP 60 peptide sequence 345-375 (P2) and Mycobacterium tuberculosis HSP 60 peptide sequence 383-402 (P6) are synthetic in that with respect to P2, the peptide sequence differs from the "natural/wild-type" of *E. coli* HSP60 such that arginine 345

(R.345) has been substituted with glycine (G.345), and with respect to P6, lysine 402 (K.402) has been substituted with glycine (G.402).

5 Sequencing algorithms can be used to measure homology or identity between known and unknown sequences. Such methods and algorithms are useful in identifying corresponding sequences present in other organisms as well as in the design of peptides of the invention. Homology or identity is often measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group,  
10 University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids, polypeptide, or peptide sequences, refer to two or more sequences or subsequences that are the same or have a specified  
15 percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

20 For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated.  
25 The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 8 to  
30 10, 10 to 20, 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of

alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol* 48:443 (1970), by the search for similarity method of person & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1997) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) or 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectations (E) of 10, and the

BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

5        The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873 (1993)). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a  
10    nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Other algorithms for determining homology or identity include, for example, in  
15    addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLOCKS IMPROVED Searcher), FASTA, Intervals  
20    & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local  
25    Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having  
30    substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human

Genome Sequencing Project (J. Roach, [http://weber.u.Washington.edu/~roach/human\\_genome\\_progress2.html](http://weber.u.Washington.edu/~roach/human_genome_progress2.html)) (Gibbs, 1995). At least twenty-one other genomes have already been sequenced, including, for example, *M. genitalium* (Fraser *et al.*, 1995), *M. jannaschii* (Bult *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), and yeast (*S. cerevisiae*) (Mewes *et al.*, 1997), and *D. melanogaster* (Adams *et al.*, 2000). Significant progress has also been made in sequencing the genomes of model organism, such as mouse, *C. elegans*, *Arabidopsis sp.* and *D. melanogaster*. Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, <http://www.tigr.org/tdb>; <http://www.genetics.wisc.edu>; <http://genome-www.stanford.edu/~ball>; <http://hiv-web.lanl.gov>; <http://www.ncbi.nlm.nih.gov>; <http://www.ebi.ac.uk>; <http://Pasteur.fr/other/biology>; and <http://www.genome.wi.mit.edu>.

Further, selection of HSP-derived peptides may be carried out by, such as, the following protocol wherein HSPs from various species are obtained using the Entrez retrieval system (<http://www.ncbi.nlm.nih.gov/Entrez/>) at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). The Entrez retrieval system allows for searching several linked publicly available databases and it provides access to the date-bases listed below. Any of these data bases may be employed in the methods of the invention, such as any of the following:

## 20 **Databases**

- PubMed: biomedical literature
- Nucleotide: sequence database (GenBank)
- Protein: sequence database
- <<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>>
- 25 -Structure: three-dimensional macromolecular structures
- Genome: complete genome assemblies
- Books: BookShelf online books
- Domains: conserved domains (CDD)
- 3D Domains: domains from Entrez Structure
- 30 -GEO: Gene Expression Omnibus
- GEO Datasets: curated GEO data sets
- Journals: journals in Entrez
- MeSH: medical subject headings
- NCBI Web Site: NCBI Web site search
- 35 -OMIM: Online Mendelian Inheritance in Man
- PMC: full-text digital archive of life sciences journal literature

- PopSet: population study datasets
  - SNP: single nucleotide polymorphisms
  - Taxonomy: organisms in GenBank
  - UniGene: gene-oriented clusters of transcript sequences
- 5    -UniSTS: markers and mapping data

Protein sequences are obtained such as the list of the retrieved HSP amino acid sequences from various species used for selecting the peptides, such as for example:

*Escherichia coli*

- 10    -hsp60: DEFINITION: GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shockprotein [Escherichia coli K12]. VERSION: NP\_418567.1 GI:16131968
- hsp70: DEFINITION: heat shock protein, chaperone, member of Hsp70 protein family [Escherichia coli K12]. VERSION: NP\_417021.1 GI:16130451
- dnaj: DEFINITION: Chaperone protein dnaJ (Heat shock protein J) (HSP40).
- 15    VERSION: P08622 GI:118719

*Homo sapiens*

- hsp60: DEFINITION: heat shock 60kDa protein 1 (chaperonin); heat shock 60kD protein 1(chaperonin) [Homo sapiens]. VERSION: NP\_002147.1 GI:4504521
- hsp70: DEFINITION: Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2).
- 20    VERSION: P08107 GI:462325
- dnaj: DEFINITION: DnaJ homolog subfamily B member 1 (Heat shock 40 kDa protein 1) (Heat shock protein 40) (HSP40) (DnaJ protein homolog 1) (HDJ-1). VERSION: P25685 GI:1706473

*Mycobacterium tuberculosis*

- 25    -hsp60: DEFINITION: heat shock 60kDa protein 1 (chaperonin); heat shock 60kD protein 1(chaperonin) [Homo sapiens]. VERSION: NP\_002147.1 GI:4504521
- hsp70: DEFINITION: Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2).
- VERSION: P08107 GI:462325

- Following retrieval of the sequences of interest, the retrieved sequences were
- 30    imported or transferred to Mac Vector 7.1.1 Trial Version to analyze the primary structure of these proteins using algorithms contained in this software package, able to originate secondary structure predictions based on the analysis of the whole protein primary sequence. Several tools were then used including the following:

**-Antigenic Index:** This index uses information that goes beyond single type analysis and the algorithm combines information from hydrophylicity, surface probability and backbone flexibility predictions along with the secondary structure predictions of other algorithms such Chou-Fasman and Gobson-Garnier. profiles originated using this algorithm identify regions of a given amino acid sequence that is predicted to be exposed to the surface and therefore that may be antigen sites. This analysis combinations is able to produce a composite prediction of the surface contour of a given protein. Regions of the protein predicted to be exposed at the protein's surface are plotted above the graph axis and vice versa non exposed regions are plotted below the graph axis (See Figs).

**-Kite-Doolittle Hydrophilicity Profile:** This algorithm profiles graphs of the local hydrophilicity of a protein along its aminoacid primary sequence. The rationale is based on the assignment of a hydropathy values to each of the 20 amino acids based on some experimental of empirical measure. Then a window of size N run along the length of the protein and each hydropathy value is summed and then divided by N to obtain an average hydrophilicity for the window. Average hydrophilicity values are then plotted to assign values above the axis denote hydrophilic regions and potentially exposed to the surface of the protein. Conversely, values below the axis indicate hydrophobic regions that tend to be buried inside the molecule or inside hydrophobic environments such as membranes. The Kite-Doolittle scale is the most commonly used hydrophathy scale and its values are assigned using a combination of water-vapor transfer free energy for amino acid side chains and the preference of amino acid side chains for interior or exterior environments, with small adjustments made to the final values based on experimental experience.

**-Surface Probability:** This algorithm predicts which regions of a protein are most likely to lie on the protein surface, based on the knowledge of which amino acids are more likely to be found on the protein surface of proteins of known structure. The algorithm employs a determination of solvent-accessible surface area of each residue. Residues were then classified as "buried" if their accessible surface area was smaller than 20 angstroms and "exposed" if the accessible surface area was larger than 60 angstroms. For each of the 20 amino acids information was compiled on what percent of the time the

amino acids were found to be exposed or buried in a sample of proteins of known atomic structure. In order to calculate the fractional surface probability for each amino acid, MacVector sums the six fractional probabilities of the amino acids in the window and divides by six fractional probabilities of the amino acids in the selected window and divides by six to yield a running average of the fractional surface probability along the length of the protein. Any value close to 1.0 at any point in the sequence of the protein would mean that the hexapeptide centered about that point is predicted to be exposed at the surface. Conversely, values close to 0.0 will mean that the hexapeptide is buried in the interior of the protein.(32,33).

**-Chou-Fasman:** This is a widely used method of secondary structure prediction based on known X-ray structures. The authors compiled statistics on the tendency of an amino acid to appear in a given secondary structure and used these statistics to assign the 20 amino acids into four classes: helix formers, helix breakers, sheet formers and sheet breakers. The algorithm then predicts the structure of proteins by locating clusters of helix- or sheet-forming residues in the amino acid sequence and applying a set of rules to determine if these clusters are significant enough to nucleate a helix or a beta sheet structure. Using the MacVector version, the prediction of conformation is treated independently of the others and each structure prediction is graphed separately.

Once the above analysis is performed, the values assigned by the three above mentioned algorithms and originated score values depending of the algorithm were visualized in the form of a Data Summary Table (DST) or in the form of a Data Plot (DP) as provided in the following examples below. Additionally, in analyzing DST and DP we selected regions of the proteins containing structures denominated  $\alpha$ -helix and  $\beta$ -turns (or loops) that are characterized by appearing on the surface of proteins and usually reversing the direction of a chain.  $\beta$ -turns refer to a section of a polypeptide connecting regions of defined secondary structure. In many proteins loops contain functional residues in that they tend to be more flexible in conformational changes than helices and sheets and therefore tend to be relevant part of binding sites. Also, in case we had a certain “core putative TLR-binding region” normally a  $\beta$ -turns predicted to be hydrophilic and surface-exposed we selected flanking regions on both sides predicted to be hydrophobic or not-surface-exposed but still predicted to be  $\alpha$ -helix. In case such prediction was not

satisfactory we made substitutions of hydrophilic amino acid with other amino acids of the appropriate characteristic.

#### EXAMPLE I: Selection of Peptide: eHSP60p1

A Data Summary Table was produced for eHSP60-P1. The primary amino acid sequence of *E. coli* HSP60 was analyzed using the combination of four different algorithms described above. The result of this analysis was displayed in a Data Summary Table I that shows region 426-444 of the primary amino acid structure of *E. coli* HSP60. Of the entire sequence analysis we selected regions that had a “Helix-Turn-Helix” predicted structure. In italics is highlighted the  $\beta$ -turn region. In plain print the flanking regions having a predicted  $\alpha$ -helix structure.

Data Summary Table I

Pos.	AA	AI	KD	hyd	SurfP	CFhlx	CFsht	CFturn	CFval	CFtype
426	L	-0.315	-0.429	0.434	1.181	0.897	0.386	0.65	H	
427	A	-0.233	0.471	0.535	1.134	0.910	0.291	0.65	H	
428	D	-0.249	0.414	0.490	1.097	0.906	0.423	0.65	H	
429	L	-0.241	0.357	0.463	1.103	0.910	0.455	0.65	H	T
430	R	-0.117	1.400	0.536	1.046	0.873	0.283	0.65	H	T
431	G	0.190	2.157	0.614	1.067	0.851	0.455	0.65	H	T
432	Q	0.208	2.157	0.614	1.067	0.851	0.315	0.65		T
433	N	0.507	3.200	0.695	1.063	0.820	0.498	0.65		T
434	E	0.481	3.057	0.666	1.054	0.787	0.294	0.65		T
435	D	0.221	2.400	0.668	1.104	0.869	0.489	0.65		T
436	Q	0.198	1.957	0.580	1.003	0.886	0.335	0.90		T
437	N	0.072	0.814	0.500	1.013	0.984	0.455	0.90		T
438	V	0.067	0.871	0.528	0.983	0.987	0.255	0.90		T
439	G	-0.293	-0.229	0.450	0.964	1.097	0.261	0.65	H	
440	I	-0.378	-0.986	0.371	0.967	1.111	0.242	0.65	H	
441	K	-0.553	-2.029	0.298	1.024	1.149	0.208	0.25	H	
442	V	-0.502	-0.786	0.405	1.031	1.077	0.259	0.00	H	
443	A	-0.531	-1.100	0.415	1.136	1.074	0.242	0.00	H	
444	L	-0.464	-0.729	0.424	1.207	1.006	0.257	0.25	H	

Next, a Data Plot was generated wherein we analyzed the same region of the *E. coli* HSP60 protein sequence originated by the same computer analysis done using a combination of the of four different algorithms described above (See Figs. 1-4). This analysis confirmed a “core sequence” including the amino acids 429-438 that was predicted to be surface exposed by the four different algorithms and matched with the “Helix-Turn-Helix” predicted structure by the Chou-Fasman analysis.

The combination of Summary Table and Data Plot provided for prediction and identification of the regions of the protein that have the highest surface exposure

prediction. For the selection of the peptides predicted to play a role in interacting with TLR on monocytes and monocytes derived DC we selected regions of the proteins which had high scores for all the three selected algorithms, *i.e.* -Antigenic Index; -Kite-Doolittle Hydrophilicity; -Surface Probability for the “core region”. Flanking regions had less surface exposure prediction score values and that satisfied the rule of “Helix-Turn-Helix” structure.

Additionally, we “refined” the selection found by the above analysis by re-analyzing the selected region, *i.e.* peptide, alone using the same algorithms and comparing these predictions with the same region within the original protein. If changes occurred in the score values we made changes of the amino acids of the flanking ends to make sure that the two predictions were as close as possible and maintained or acquired the “Helix-Turn-Helix” structure. This refinement produced the affect on Antigenic Index, hydrophilicity, Surface Probability, and Chou-Fasman as shown in Figures 5-8 wherein region 426-444 of the primary amino acid structue of *E. coli* HSP60 is reanalyzed using the combination of four different algorithms described above.

As an example of such refinement, the invention peptides include derivative peptides of wild-type peptides such as P2 and P6 disclosed in Table I. These single amino acid changes were brought about because in the computer analysis these substitutions generated a “predicted-structure” more likely to be surface-exposed.

Further refinement of the peptide selection was carried out (based on computer models if available). For example, a three dimensional computer model of *E. coli* HSP60 protein was used to examine the position of the P1 peptide. Selected regions were originated using as source the structural coordinates of the crystallized *E. coli* protein Groel (otherwise called HSP60) and visualized using Cn3D4. This software is a visualization tool for biomolecular structures, sequences, and sequence alignments and is publicly available at: <<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>>. In yellow is highlighted the region 426-444 of the *E. coli* HSP60 protein. It can be noted that the region 426-444, as predicted, is surface exposed (See Figure 9).

EXAMPLE 2: Effect of HSP-derived peptides on immune modulation

HSP-derived peptides that bind TLR receptors can affect expression of TLR2, TLR4 receptor and secretion of TNF-alpha and IL-6 proinflammatory cytokines, on monocyte-derived dendritic cells. Here, we show that human heat shock protein 60 (hHSP60) elicits a potent pro-inflammatory response in cells of the innate immune system and therefore has been proposed as a danger signal of stressed or damaged cells. Previous work showed that mouse or human macrophages, as well as endothelial or smooth muscle cells, were found to elicit a pro-inflammatory response when incubated with recombinant hHSP60. The response included the up-regulation of adhesion molecule expression and the release of inflammatory mediators such as IL-6 and TNF-alpha. Since autologous hsp60 may be aberrantly expressed on the cell surface in response to stress and will be set free from the cell interior during necrosis, these findings point to a role of hHSP60 in initiating or sustaining Th1- dependent tissue inflammation. Interestingly, microbial HSP60/65 also induces a pro-inflammatory response in innate immune cells suggesting that damaged autologous cells and microbial pathogens may alert innate immunity via the same recognition system. TLR4 receptor has been identified to mediate HSP60 signaling while TLR2 appears to be important in LPS binding and signaling. We decided to investigate if peptides derived from phylogenetically separate hsp60 species would interact with innate immune cells via different recognition pathways as Tlr2 and Tlr4 As shown in Figure 10A-D, monocyte-derived dendritic cells respond to the different peptides with a kinetics that revealed peak levels of expression at 6 hours after stimulation. P1, P3, and P4 were able to induce, at various levels, expression of TLR2 and TLR4 receptor. Also TNF-alpha and IL-6 cytokine secretion was measured with P1, P3 and P4. These data indicate correlation between receptor expression and pro-inflammatory cytokines secretion. TNF-alpha and IL-6 production was induced with P2 at 6 hrs (Fig. 10C-D) but very low level of Tlr2 and Tlr4 receptor expression was detected (Fig. 10A-B). This result may be due to a different activation pathway by the P2 peptide compare to the previous peptides. Interestingly, P1 was affecting TLR2 and TLR4 expression at 48 hrs and not at 24 hrs (Fig. 10A-B), while P4 was giving TLR4 expression up to 24 hrs after stimulation (Fig. 10B) but no cytokine secretion was measurable. As a positive control, HSP60 was used

for all parameters analyzed (Figs. 10A-D) while LPS was used for TNF-alpha and IL-6 induction.

### *Materials and Methods*

#### 5 Monocytes and DC preparation

PBMC were derived by Ficoll-Hypaque density gradient centrifugation from buffy coats of healthy blood donors obtained from the San Diego Blood Bank. CD14-positive monocytes were enriched to 93% purity by Percoll density gradient centrifugation. DC were generated by previously isolated monocytes in IMDM medium  
10 supplemented with 1% human AB serum, GM-CSF and IL-4 for 6 days, replacing 50% of the culture medium every other day.

#### Induction of TLR2, TLR4 and cytokine release from monocyte-derived dendritic cells

DC (1x10<sup>6</sup> cells/well) were stimulated with the different peptides and as a control HSP and LPS were used. Cells were harvested respectively at 6, 24, 48, and 72 hours.

#### 15 Flow cytometry assay.

Specific cell surface staining was performed using FITC, PE and Cy-conjugated antibody (Ab) in saturating amounts. Specific isotype controls were used for each sample. Anti-TLR-2, anti-TLR-4, anti-CD86, anti-CD83, anti-HLA-DR, anti-TNF- $\alpha$  and anti-IL-6 Abs were used. Results were expressed as the percent of positive cells for a  
20 given marker. The relative percent of fluorescence intensities was calculated as the value of the percent of fluorescence intensities of the respective marker Ab minus the specific isotype control and the values obtained with non-stimulated cells.

EXAMPLE 3: Effect of HSP-derived peptides on secretion of TNF-alpha from a mouse  
25 macrophage cell line, RAW264.7.

As discussed above, we wanted to investigate if peptides derived from phylogenetically separate hsp60/70 species would interact and elicit an inflammatory response in a mouse monocyte/macrophage cell line. As shown in Figure 11, RAW264.7 cells respond to the different peptides with a kinetics that revealed peak levels of  
30 expression at 72 hours after stimulation. P10 (hsp70 human derived peptide) and P3, P4 (hsp60 human derived peptide) are inducing a very strong TNF- $\alpha$  secretion. Also, P1 and

P2 (HSP60 *E. coli*-derived peptide) and P5, P6 (hsp60 *Mycobacteria*-derived peptide) are inducing a very comparable TNF- $\alpha$  secretion. We conclude that phylogenetically separate hsp60 / 70 can induce an inflammatory response, as indicated by the medically relevant mouse cell line model.

5

#### EXAMPLE 4: Diagnostic Application

In this example, the peptides of the invention are used for diagnosis of patients with Autoimmune Diseases. Specifically, a panel of the selected/identified HSP-derived peptides are used to screen patient PBMC-derived Monocytes, Macrophages and  
10 Monocyte derived-dendritic cells as well as other immune cells expressing TLR. Such screening can be used to identify specific TLRs that can be then “targets” for anti-inflammatory therapy. Such TLRs can be disease specific. This then provides for autoimmune specific/tailored immune intervention based on the inhibition of innate immune mediated proinflammatory pathway activation.

15 Natural as well as synthetic antigen-specific therapeutics derived from TLR binder peptides can be then used to interfere/inhibit the binding of HSP and their derivatives to TLRs and therefore inhibiting or interfering with the resulting triggered inflammatory response in a disease-specific fashion.

#### 20 EXAMPLE 5: Therapeutic Application:

Therapeutic applications comprise three general medical areas including cancers, infectious diseases and immunodeficiencies and in each of these areas, the HSP-derived peptide sequences of the invention may be identified for interacting with specific TLR allowing additionally the capability of designing peptides or peptidomimetics which bind  
25 directly to TLR and induce a pro-inflammatory response. Clinical applications range from Cancer (systemic or local) to infectious diseases (including granuloma) and immunodeficiencies. In another aspect, clinical applications include use of the peptides as adjuvants in vaccine formulations against a whole range of infectious disease and cancer. In yet another aspect, the peptides of the invention can be applied to autoimmune  
30 diseases, for example, the HSP-derived peptide sequences can be identified and designed or peptidomimetics created for interacting with or binding directly to specific TLR to

avoid inducing proinflammatory responses. In this instance, the invention peptides are contemplated to act in the form of, for example, molecular competitor/inhibitor of natural HSP, or triggers of DC-2-type responses (tolerogenic) by acting on and triggering different activation pathways. Further, in this example, identified HSP-derived peptide sequences interacting with specific TLR can be used for the development/identification of natural as well as synthetic antigen-specific therapeutics able to interfere/inhibit the binding of HSP and their derivatives to TLRs and therefore inhibiting or interfering with the resulting triggered inflammatory response.

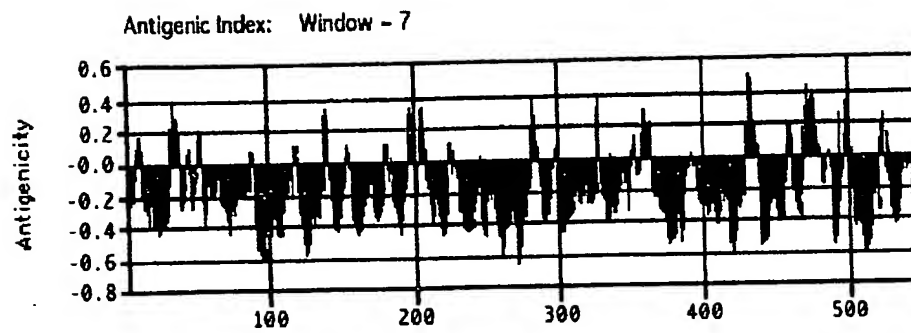
Inhibition or interference with the inflammatory response include the inhibition of all or part of the proinflammatory mediators such as TNF- $\alpha$  or IL-6 and of the Th1-promoting cytokines IL-12 and IL-15 and possibly other cytokines and mediators secreted as a results of interaction between both bacterial and human HSPs and TLR expressed on Monocytes, Macrophages and Monocyte-derived Dendritic cells as well as other immune cells expressing TLR.

Natural as well as synthetic antigen-specific therapeutics derived from the HSP-derived peptides able to interfere/inhibit the binding of HSP and their derivatives to TLRs can be preferably administered. Further, peptido-mimetics synthetic molecules able to block specific TLR involved in the induction of proinflammatory cytokine secretion can be designed. Further still, immunization with TLR identified in the screening assay can be accomplished to bring about lowering of inflammatory responses.

## ABSTRACT

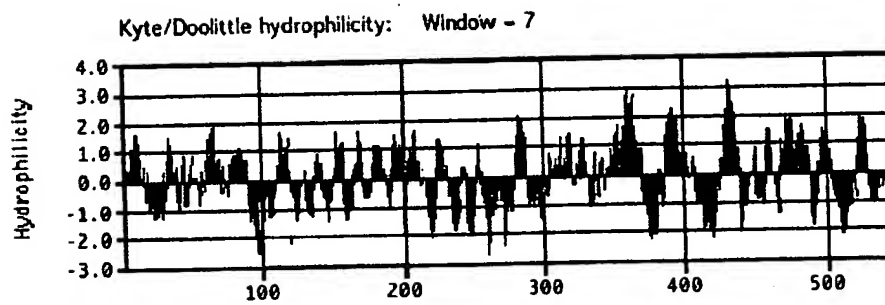
Toll receptor-binding peptides are disclosed. Also disclosed are methods of using  
5 such peptides in modulating immune responses in immune-mediated diseases. Further  
disclosed are in silico methodologies for identifying TLR-binding peptide motifs.

Figure 1



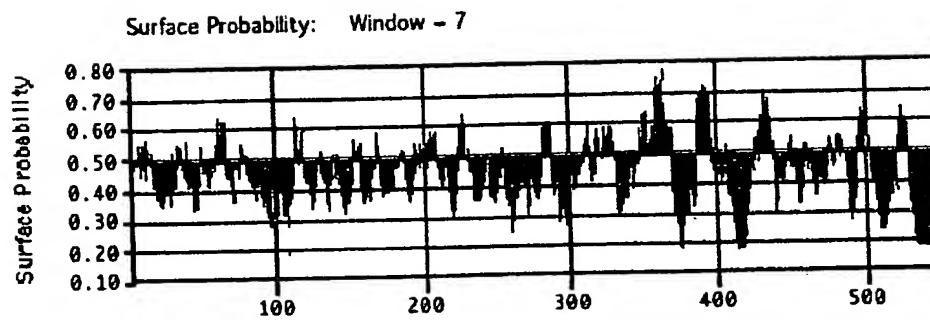
5

Figure 2



10

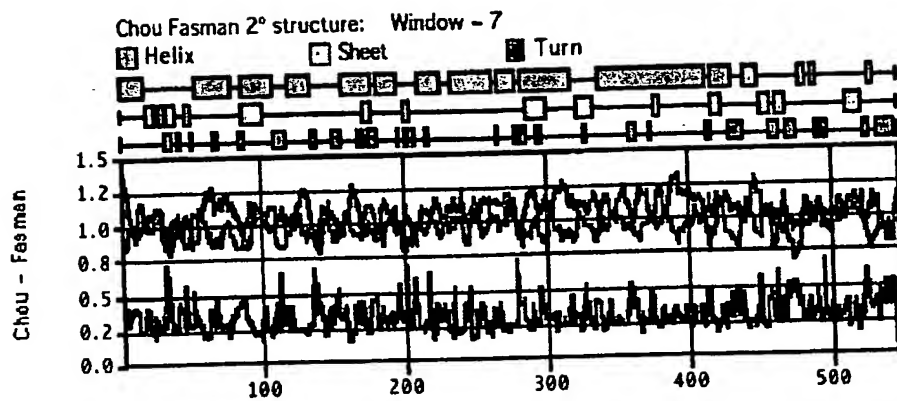
Figure 3



15

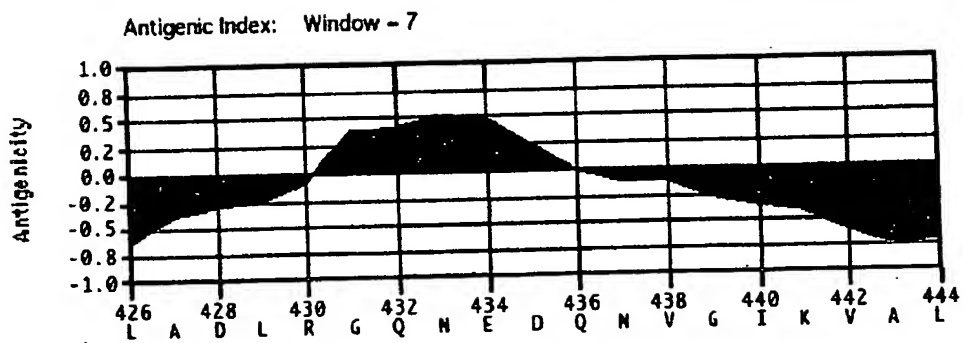
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Figure 4



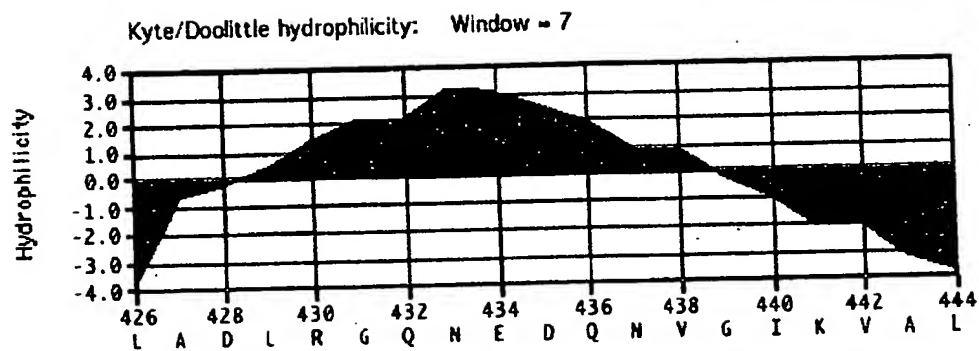
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Figure 5



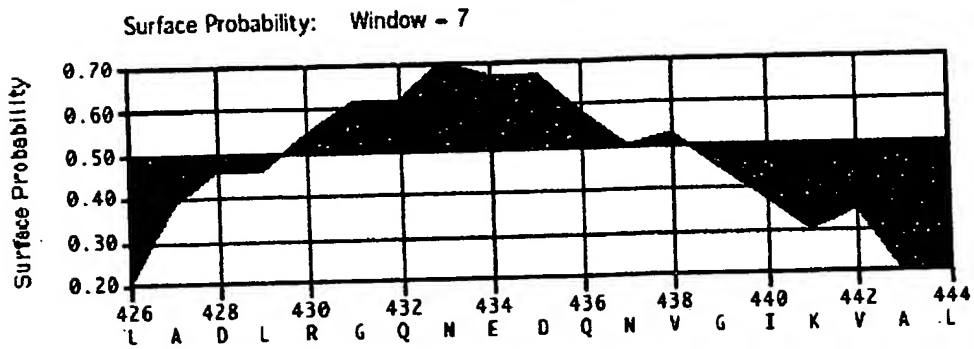
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Figure 6



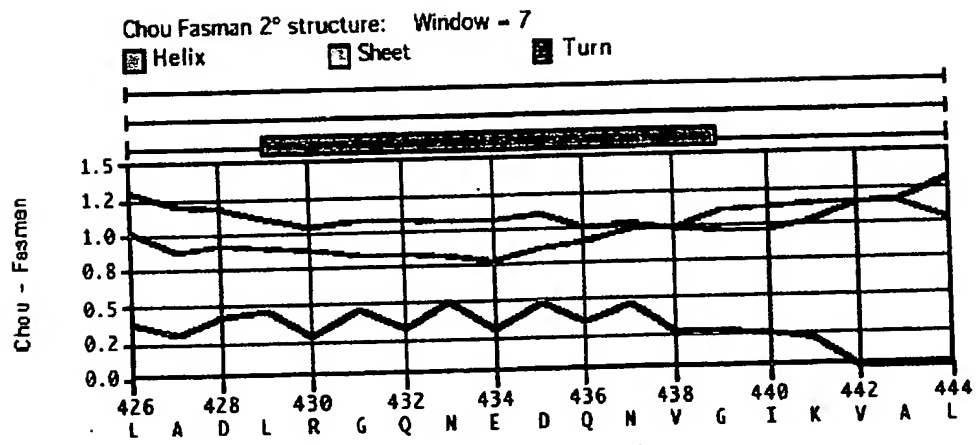
15

Figure 7



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Figure 8



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Figure 9

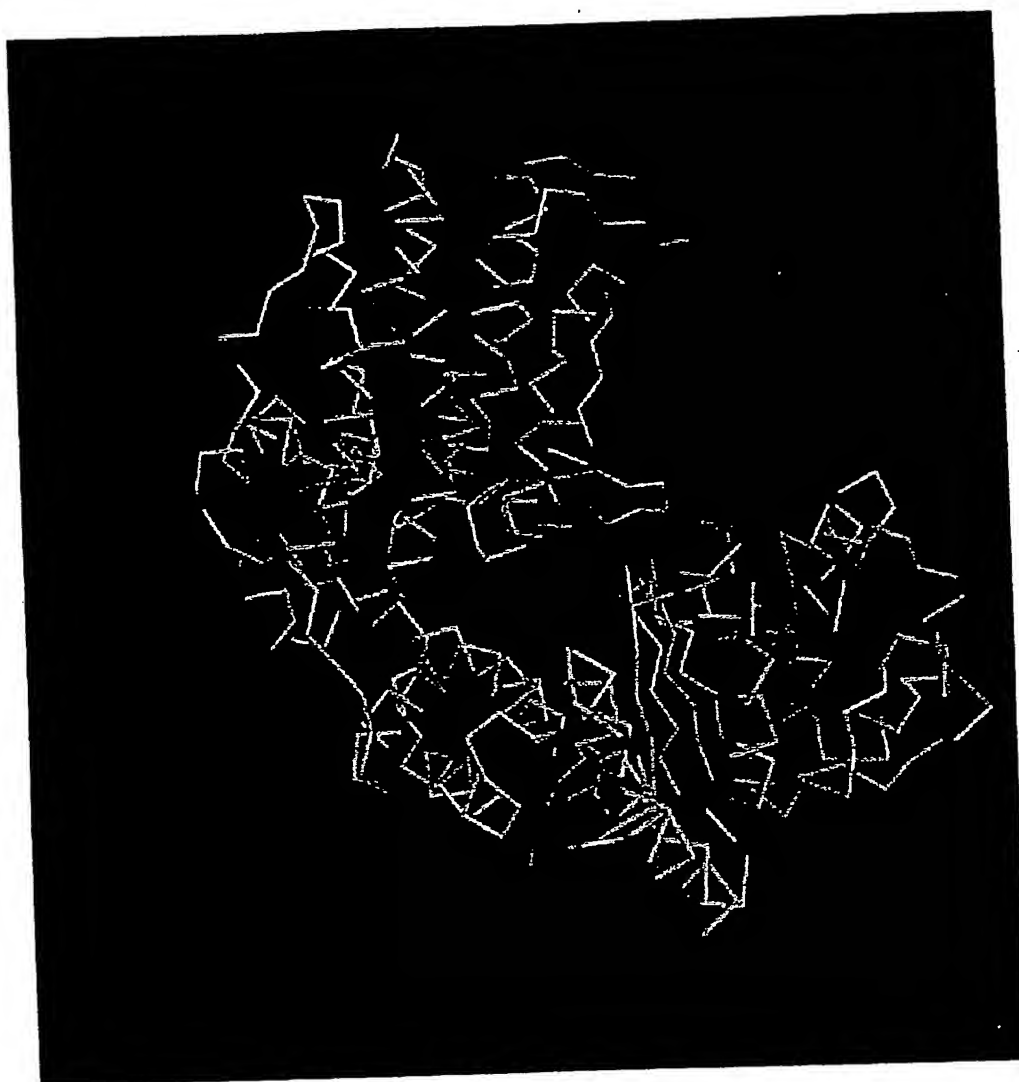
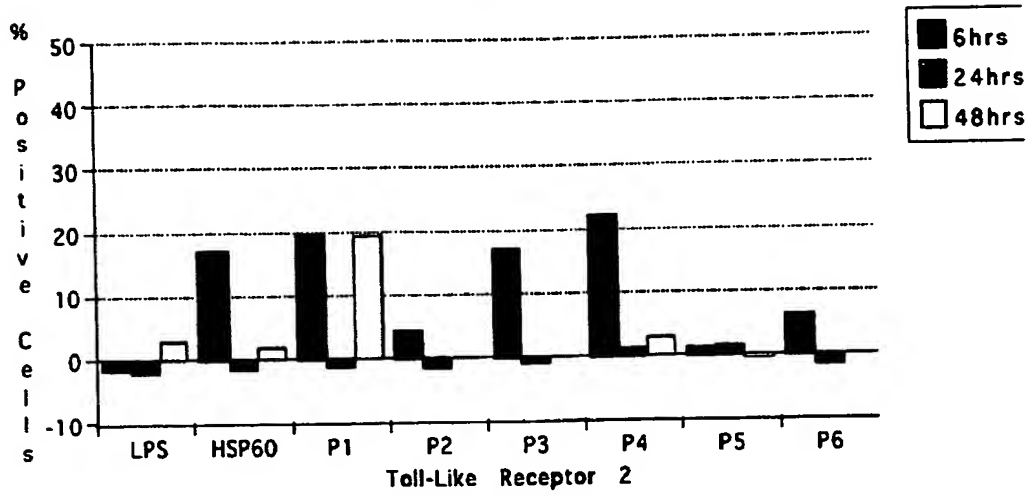
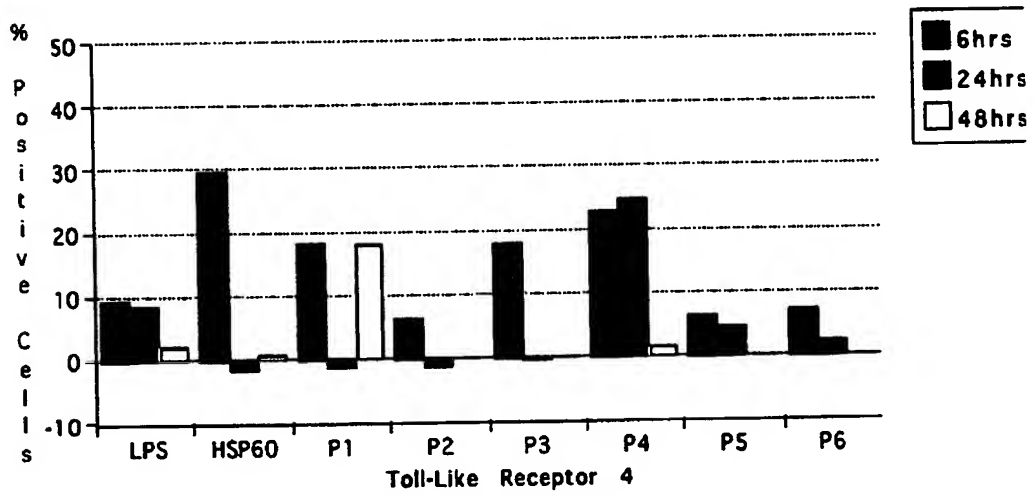


Figure 10A



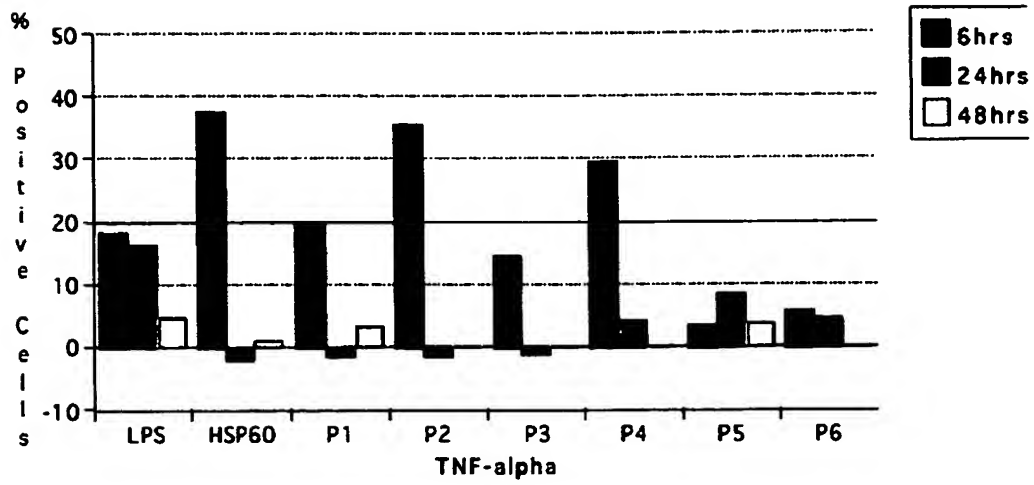
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Figure 10B



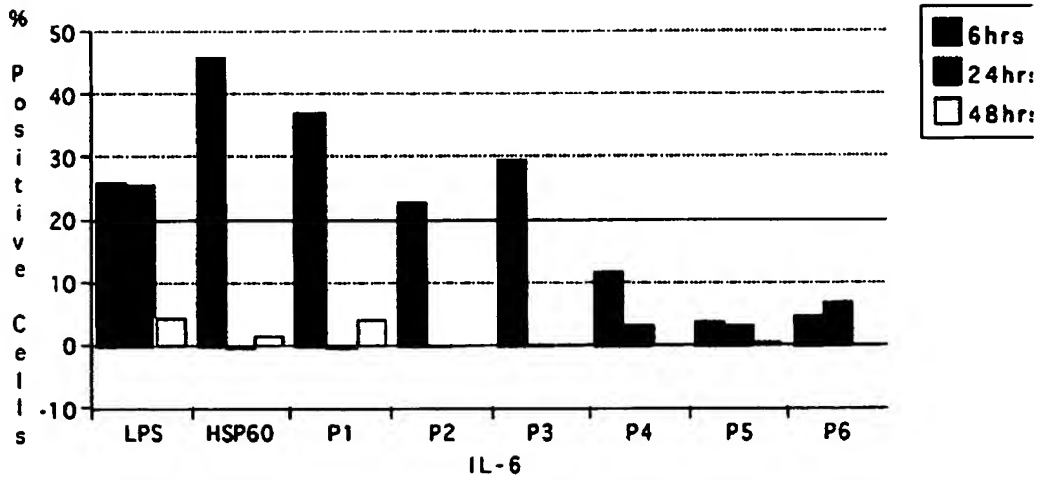
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Figure 10C



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Figure 10D



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Figure 11

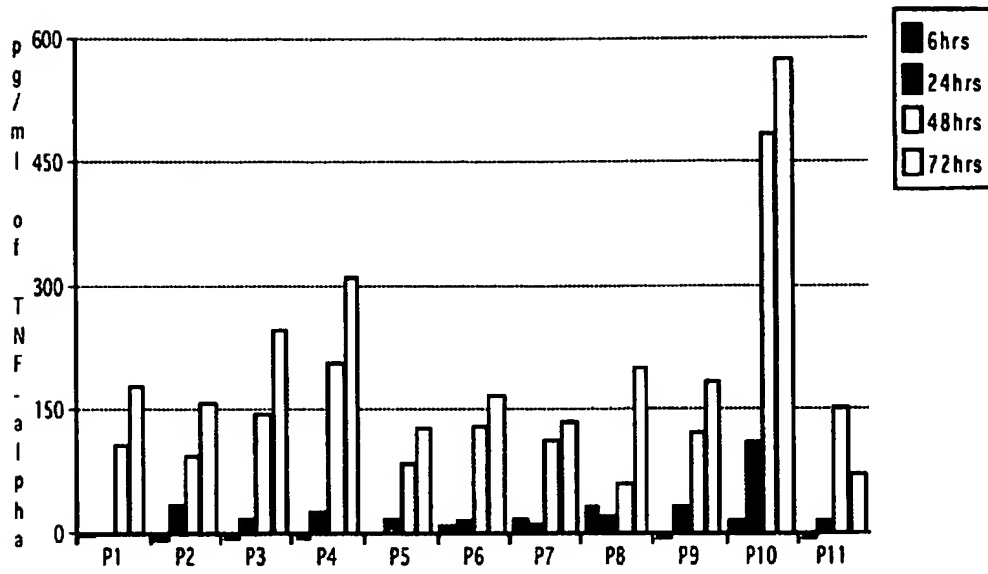


Figure 12

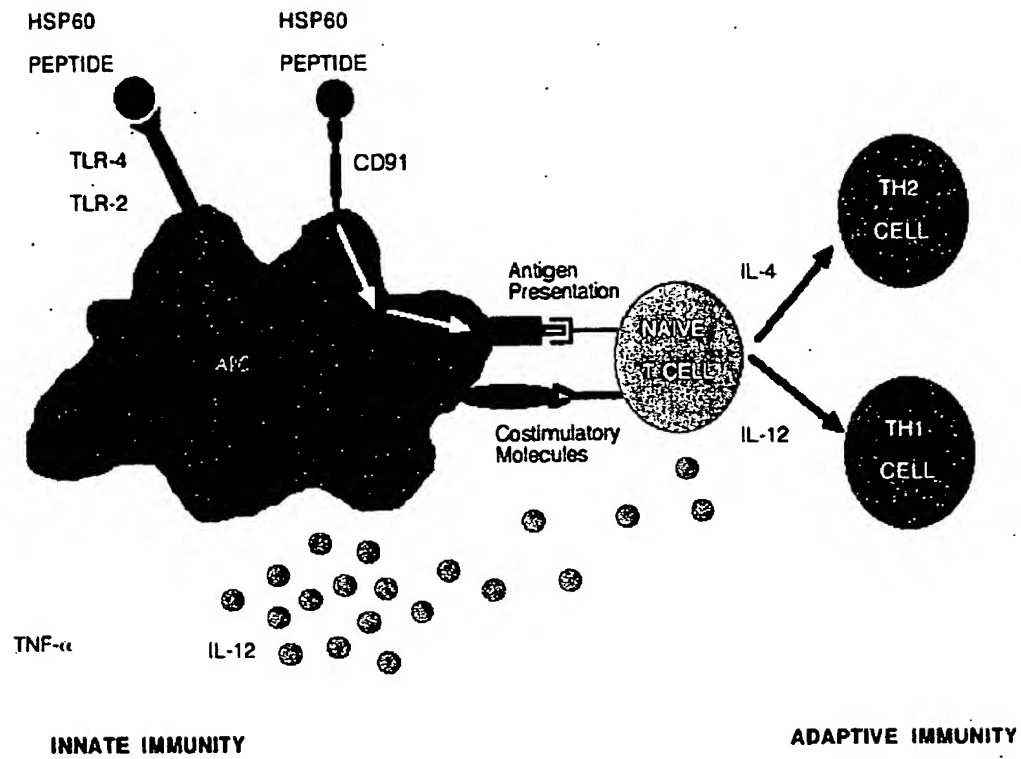


Figure 13

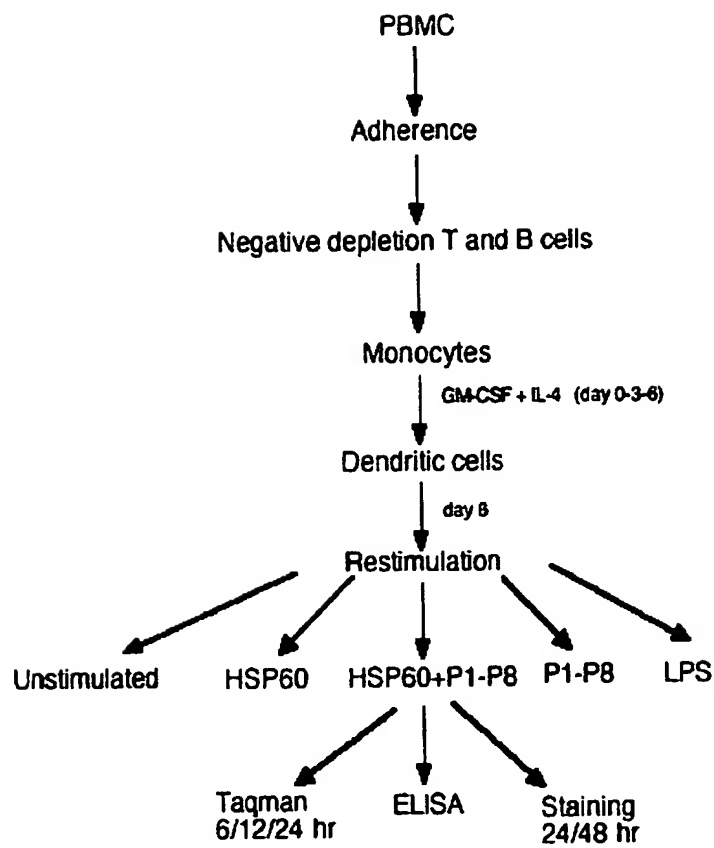


Figure 14

TNF- $\alpha$  production of Dendritic Cells measured with Real Time PCR

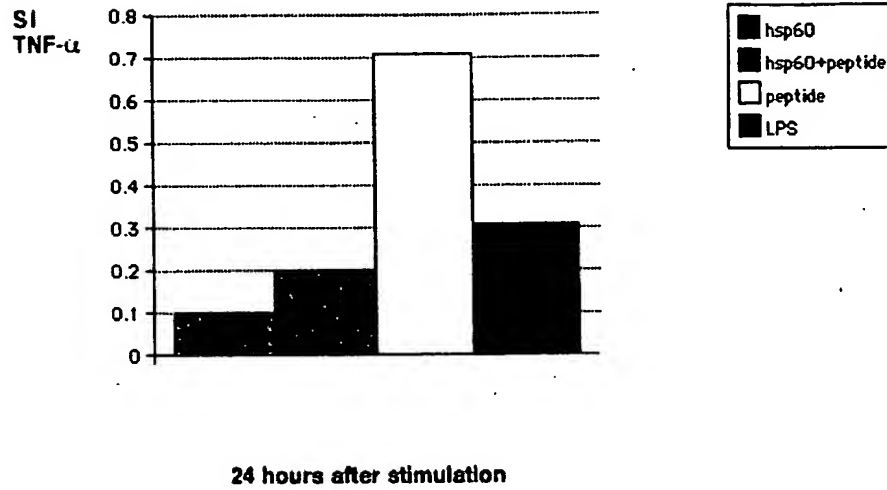
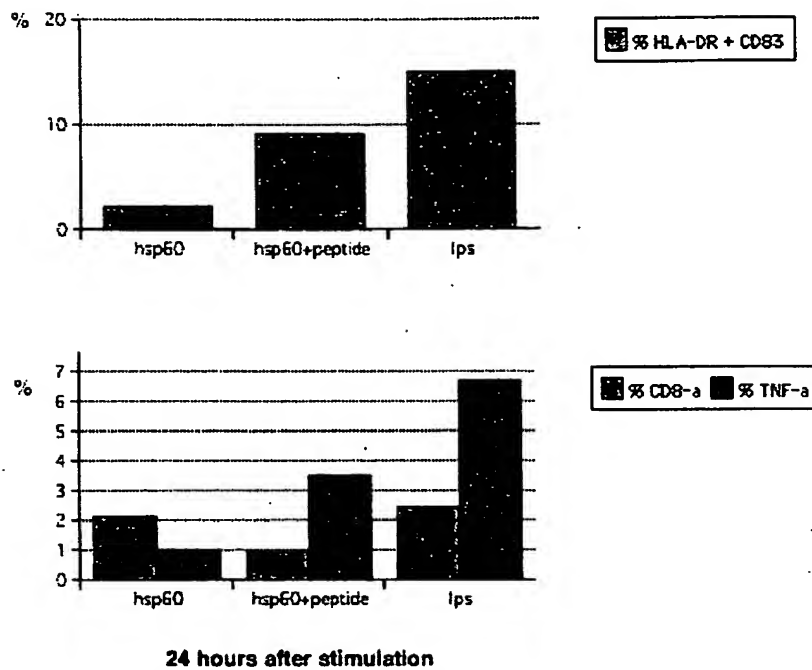


Figure 15A (top) and B(bottom)  
 Percentage of CD8-a positive cells and TNF-a production by Dendritic cells  
 (CD83+ HLA-DR+) measured with staining



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